Structure and Expression of Fibulin-2, a Novel Extracellular Matrix Protein with Multiple EGF-like Repeats and Consensus Motifs for Calcium Binding

Te-Cheng Pan,* Takako Sasaki,‡ Rui-Zhu Zhang,* Reinhard Fässler,‡ Rupert Timpl,‡ and Mon-Li Chu*

*Departments of Biochemistry and Molecular Biology and Dermatology, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107-5541; and ‡Max-Planck-Institut für Biochemie, D-82152 Martinsried, Federal Republic of Germany

Abstract. A new protein, fibulin-2, was predicted from sequence analysis of cDNA clones obtained from a mouse fibroblast library. This protein consists of a 1195-residue polypeptide preceded by a 26-residue signal peptide. The COOH-terminal region of 787 amino acids contained three anaphylatoxin-related segments (domain I), 11 EGF-like repeats (domain II), 10 of which had a consensus motif for calcium-binding, and a 115-residue globular domain III. Except for two additional EGF-like repeats, this COOH-terminal region showed 43% sequence identity with the previously described fibulin-1 (BM-90). The NH_2-terminal 408 residues, unique to fibulin-2, showed no sequence homology to other known proteins and presumably form two additional domains that differ in their cysteine content. Recombinant fibulin-2 was produced and secreted by human cell clones as a disulfide-bonded trimer. Rotary shadowing visualized the protein as three 40–45 nm long rods which are connected at one end in a globe-like structure. No significant immunological cross-reaction could be detected between fibulin-1 and fibulin-2. Production of the fibulin-2 was demonstrated by Northern blots and radioimmunoassay in fibroblasts but not in several tumor cell lines. Together with the observation that the serum level of fibulin-2 is 1,000-fold lower than that of fibulin-1, the data indicate that these two isoforms are not always coordinately expressed. This is also suggested by Northern blots of tissue mRNAs and by immunofluorescence localizations using mouse tissues. The latter studies also demonstrated an extracellular localization for fibulin-2 in basement membranes and other connective tissue compartments.

M OST extracellular matrix proteins have mosaic structures (Bork, 1991; Engel, 1993) and frequently possess ~50-residue repeats homologous to EGF. These repeats usually contain three or, as with laminin and perlecan, four disulfide bridges stabilizing a tightly folded structure (Baron et al., 1991; Selander-Sunnerhagen et al., 1992). As shown for laminin (Beck et al., 1990; Engel, 1993) and tenascin (Spring et al., 1989), such repeats can form rodlike structures if they occur in multiple consecutive copies, and thus may serve as structural or spacing elements. An additional function is their binding of calcium, indicated by a special sequence motif identified so far in more than 150 EGF-like repeats (Rees et al., 1988; Handford et al., 1991; Selander-Sunnerhagen et al., 1992). This consensus sequence has for example been found in the basement membrane proteins nidogen (Mann et al., 1989) and fibulin/BM-90 (Argraves et al., 1990; Pan et al., 1993), and is especially abundant in the microfibrillar protein fibrillin (Lee et al., 1991; Maslen et al., 1991). Studies with coagulation factors have shown that calcium-binding affinities of such repeats are in the millimolar range (Rees et al., 1988; Persson et al., 1989; Handford et al., 1990), suggesting that modulation of binding can occur in the extracellular space. Of particular interest, although less well studied, is the participation of EGF-like repeats in the interactions with extracellular matrix proteins and cellular receptors. This was shown for a single repeat of the laminin B2 chain which binds nidogen (Mayer et al., 1993) while other repeats may be responsible for laminin's mitogenic activity (Panayotou et al., 1989). The biological relevance of these structures has been emphasized by mutations found in fibrillin that cause Marfan syndrome (Dietz et al., 1991, 1992; Lee et al., 1991) and mutations in the neurogenic *Drosophila* protein notch which are lethal (Kelley et al., 1987).

Recent cDNA sequence analysis of human and mouse

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fibulin-1 (Argraves et al., 1990; Pan et al., 1993) has shown that the major central domain of the protein consists of nine EGF-like repeats, with eight of them possessing the consensus sequence for calcium binding. This protein was originally described as integrin-binding fibulin from human placenta (Argraves et al., 1989) and as BM-90 from a basement membrane–producing mouse tumor (Kluge et al., 1990). Preliminary electronmicroscopical data suggested a rodlike structure for fibulin-1 (Kluge, 1990), consistent with the sequence predictions. Calcium binding to fibulin-1 is apparently required to mediate its binding to laminin and nidogen (Pan et al., 1993). Other interactions were observed with fibronectin and with fibulin-1 itself but were not dependent on calcium (Balbona et al., 1992; Pan et al., 1993). Little else is known about possible functions except that fibulin-1 shows prominent expression at sites of epithelial endothelial-mesenchymal transitions during embryonic development (Spence et al., 1992; Zhang et al., 1993).

In our sequence analysis of mouse fibulin-1 (Pan et al., 1993), several similar cDNA clones were obtained that suggested the existence of a second isoform. Completion of the sequence of these clones now predicts a novel and longer protein, fibulin-2. This also suggests that both isoforms are members of a new family of genetically related extracellular matrix proteins. Recombinant expression of fibulin-2 demonstrated its assembly into oligomers consisting of three identical subunits, while fibulin-1 consists of monomers. Additional differences between the fibulin isoforms were noted in their expression patterns in cultured cells and tissues, suggesting that they may have different biological functions.

Materials and Methods

Isolation and Sequencing of cDNA Clones

A mouse fibroblast cDNA library was prepared from 5 μg of poly A-enriched RNA using reagents from a cDNA synthesis kit (Pharmacia Inc., Piscataway, NJ) and a λZAP II cloning kit (Stratagene Inc., La Jolla, CA). The unamplified cDNA library (~5 × 10⁵ clones) was screened with a 650-bp human fibulin-1 cDNA prepared by the polymerase chain reaction based on published sequences (Argraves et al., 1990; Pan et al., 1993). The screening was performed under low stringency hybridization conditions (6 × SSC at 55°C) (Sambrook et al., 1989). Several positive clones with insert sizes from 1.2 to 4.4 kb were characterized by dideoxynucleotide DNA sequencing (Sanger et al., 1977). The DNA sequence was compared to sequences in the databank using the FASTA computer program (Pearson and Lipman, 1988).

Northern Blot Analysis

Total RNA was prepared from mouse fibroblasts using the acid guanidine thiocyanate/phenol/chloroform extraction procedure (Chomcynski and Sacchi, 1987). Total RNAs were separated on a 1% agarose gel containing 6% formaldehyde and transferred to the Hybond N nylon membrane (Amersham Corp., Arlington Heights, IL). A Northern blot filter containing poly A-rich RNA from various mouse tissues was obtained from Clontech Laboratories (Palo Alto, CA). The RNA filters were hybridized with nick-translated fibulin-1 (MK24) (Pan et al., 1993), fibulin-2 (MK9), or human actin (American Type Culture Collection, Rockville, MD) cDNA probes under stringent conditions as described (Sambrook et al., 1989).

Expression Vector, Transfections, and Cell Culture

A full-length fibulin-2 cDNA clone (MK9, 4,377 bp in pBluescript SK-) was linearized at the unique BglII site in the 3'-untranslated region of the cDNA insert, and then blunt-ended by filling in using the Klenow fragment of DNA polymerase. The cDNA insert was released by digestion with NotI in the

polylinker of the pBluescript vector. The eukaryotic Rc/CMV expression vector (Invitrogen, San Diego, CA) was linearized at the XbaI site in the polylinker region, blunt-ended, and then digested with NotI, whose site occurs 5' to the XbaI site in the polylinker region. The resulting vector was ligated with the cDNA insert, yielding an expression plasmid pCMK9 comprising 50 bp of the 5'-untranslated region, 3,663 bp of the entire coding region and 370 bp of the 3'-untranslated region.

Human embryonic kidney 293 cells (American Type Culture Collection) were cotransfected with pCMK9 and plasmid pSV₂pac in order to generate stably transfected cell clones (Nischt et al., 1991). Those clones, which produced and secreted fibulin-2, were identified by SDS electrophoresis of serum-free culture medium examined under reducing conditions. Serum-free culture medium was then prepared on a large scale (0.5-1 liter) by established protocols (Fox et al., 1991; Nischt et al., 1991).

Protein Purification

Fibulin-2 was precipitated from serum-free culture medium (~0.5 liter) by adding solid ammonium sulfate to 50% saturation (overnight, 4°C). The precipitate was then dissolved in ~5 ml of 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, containing protease inhibitors and passed over a Sepharose CL-6B column (3 × 130 cm) equilibrated in the same buffer (Paulsson et al., 1987a). Pooled fractions containing the protein were subsequently dialyzed against 2 M urea, 0.02 M bis-Tris-propane-HCl, pH 6.5, 5 mM EDTA and chromatographed on a Mono Q column (HR5/5; Pharmacia) which was eluted with a linear NaCl gradient (0–0.6 M NaCl, 35 ml) prepared in the same buffer. If a final purification step was required, chromatography on a Superose 6 column (HR 16/50; Pharmacia Inc.) in 0.2 M ammonium acetate, pH 6.8, was used. Samples were dissolved in neutral buffer and stored at -20°C. Reduction and alkylation in 6 M guanidine-HCl followed a procedure previously reported (Kluge et al., 1990).

Fibulin-1 (BM-90) was prepared either from a mouse tumor (Kluge et al., 1990) or obtained as a recombinant product based on cDNA encoding the splice variant D (Pan et al., 1993) following a protocol as outlined above (Sasaki, T., M.-L. Chu, and R. Timpl, unpublished data). Recombinant mouse nidogen (Fox et al., 1991) and human BM-40 (Nischt et al., 1991) were prepared as previously described. Laminin-nidogen complex and the heparan-sulfate-proteoglycan perlecan were purified from a mouse tumor (Paulsson et al., 1987a and b). Human plasma fibronectin (Behringwerke Ag, Marburg Lahn, Germany) and vitronectin (Yatohgo et al., 1988) were obtained by chromatography on heparin-Sepharose.

Analytical Methods and Rotary Shadowing

PAGE (3–10% gradient gels) in the presence of SDS followed established protocols. Runs were calibrated with protein standards used in nonreduced or reduced form. Protein samples were hydrolyzed with 6 M HCl (16 h, 110°C) for the determination of amino acid composition and protein concentrations using an LC 3000 analyzer (Biotronik). Amino acid sequences were determined by a 473A gas-phase sequencer (Applied Biosystems, Inc., Foster City, CA) following the manufacturer's instructions. Rotary shadowing and EM of proteins followed previously described methods (Paulsson et al., 1987a and b).

Immunological Methods

Immunization of rabbits, ELISA, and radioimmuno-inhibition assays followed standard procedures (Timpl, 1982). Affinity-purified rabbit antibodies against mouse fibulin-1 (BM-90) were those described previously (Kluge et al., 1990). Most tissues used for indirect immunofluorescence were obtained from a 4-wk-old female BALB/c mouse. The skin specimen was isolated from the abdominal region of a 1-day-old BALB/c mouse. All tissue specimens were frozen by liquid nitrogen and cryosections (~6 μm) were prepared on a Reichert-Jung cryostat (Leica, Inc., Deerfield, IL). Sections were fixed in acetone for 10 min at -20°C and washed in PBS, pH 7.2. Antibodies against fibulin-1 (1:20) and fibulin-2 (1:150) were diluted as indicated in PBS containing 1% ovalbumin. Normal rabbit serum (1:150) served as a negative control. Fluorescence labeling was performed with FITC-conjugated swine anti-rabbit immunoglobulins (DAKOPATTS, Copenhagen, Denmark). All the fluorescent specimens were mounted in 10% glycerol containing 1 mg/ml β-phenylenediamine (Johnson et al., 1982). Microscopy was carried out with a Axiophot fluorescence microscope (Zeiss, Oberkochen, FRG).

Results

Cloning and Sequence Analysis of Fibulin-2

Screening of a mouse fibroblast cDNA library with a human fibulin-1 cDNA probe under low stringency conditions yielded two groups of homologous cDNA clones. One group encoded two different splice variants of mouse fibulin-1 (Pan et al., 1993). The second group encoded a larger, novel protein referred to in the following discussion as fibulin-2. The largest fibulin-2 cDNA clone (MK9, 4.4 kb) was used to determine the whole nucleotide sequence (Fig. 1).

The nucleotide sequence of clone MK9 predicted an open reading frame of 3,633 bp, 50 bp of 5'-untranslated region, and 664 bp of 3'-untranslated region (Fig. 1). Applying the
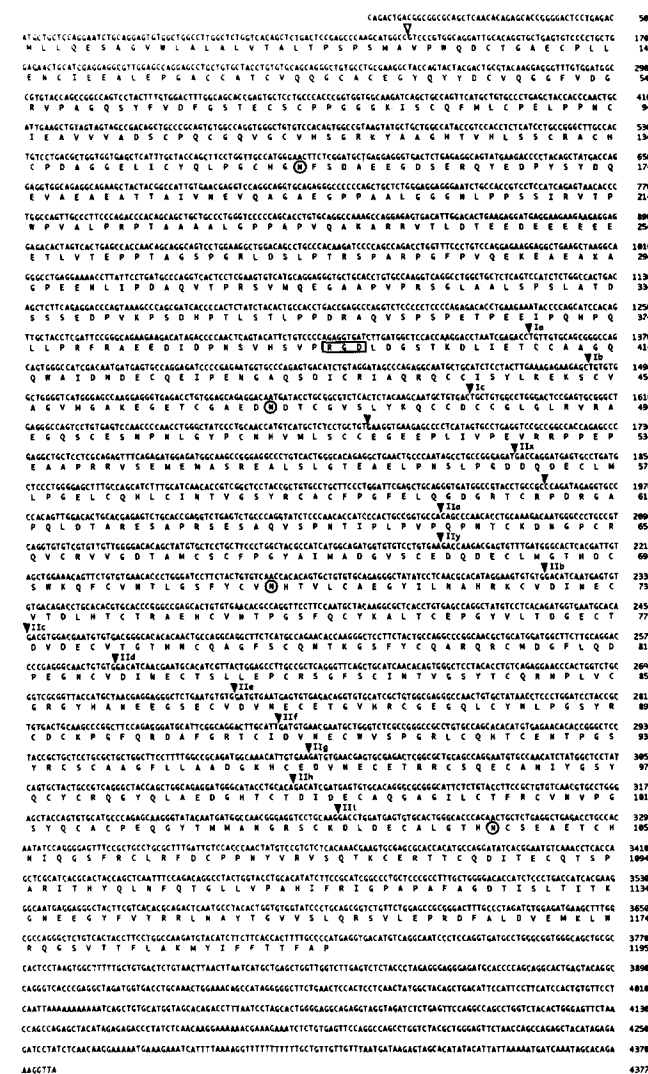
(-3, -1) rule (von Heijne, 1985), a 26-residue signal peptide and 1195 residues for the mature protein were predicted. Fibulin-2, therefore, exceeds the size of mouse fibulin-1 variant C (Pan et al., 1993) by 576 residues. The region of similarity between the two fibulins is restricted to the 3'-half of fibulin-2 (60% nucleotide identity). The predicted amino acid sequence shows that fibulin-2 is rich in cysteine (107 residues), and contains four N-linked oligosaccharide acceptor sites scattered throughout the molecule (Fig. 1). A single RGD sequence (positions 395-397) suggests a cell-adhesive site. A highly acidic region (9 Glu, 1 Asp) is found at positions 246-255. A similar acidic sequence followed later by RGD has been described for bone sialoprotein (Oldberg et al., 1988).

Four smaller fibulin-2 cDNA clones were also partially sequenced. These clones (2.5-3 kb) showed a gap in the nucleotide sequence (positions 2175-2316) corresponding to the predicted subdomain IIy (Fig. 2), suggesting a modification by alternative splicing. Four fibulin-1 splice variants (A-D) have been described (Argraves et al., 1990; Pan et al., 1993) which differ at their 3' ends and the common point of deviation would correspond to nucleotide position 3669 in fibulin-2. The examination of 6 fibulin-2 cDNA clones encompassing this region, however, did not reveal any sequence difference.

Domain Structure and Comparison with Fibulin-1

The fibulin-2 sequence predicts a multidomain structure (Fig. 2). The NH2-terminal 408 residues (domain N) are unique to fibulin-2 and show no sequence similarity to fibulin-1 or to any other protein sequence found in the EMBL data bank. 22 cysteines are clustered in the first 150 residues of domain N, which suggests that the whole structure is folded into two different subdomains, Na (Cys-rich) and Nb (Cys-free). We could neither detect any compelling internal homology in the cysteine patterns of domain Na nor any similarity to cysteine arrays in other proteins.

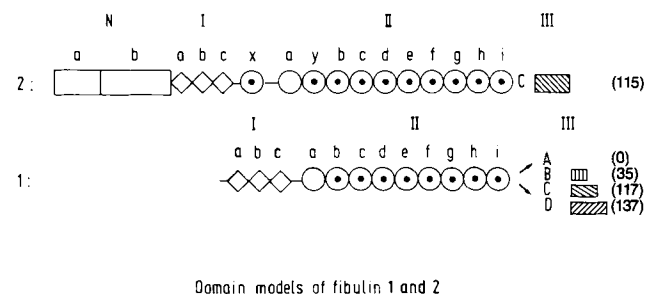The remaining structure of fibulin-2 is much more similar

Figure 1. Nucleotide sequence of fibulin-2 cDNA clone MK9 and deduced amino acid sequence. The signal peptidase cleavage site is indicated (∇); amino acid numbering starts after this site. Full arrow heads denote the beginning and ending of various domains and subdomains (see Fig. 2). Asparagines identified as potential N-glycosylation sites are circled and an RGD sequence is boxed. These sequence data are available from EMBL/GenBank/DDBJ under accession number X75285.

Figure 2. Domain model of fibulin-2 (*2*) and comparison with that of fibulin-1 (*1*) starting with the NH2 termini at the left side. Diamonds in domain I indicate three motifs related to anaphylatoxins. Circles in domain II represent EGF-like repeats and those with a dot possess a consensus motif for calcium binding. The unique globular domain N consists of a Cys-rich (*a*) and a Cys-free (*b*) segment. The COOH-terminal domain III exists in fibulin-1 in four alternatives, *A-D* (amino acid numbers in brackets), while only a homolog to variant C has been detected in fibulin-2. The domain model for fibulin-1 is based on previous data (Argraves et al., 1990; Pan et al., 1993).

Domain I

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           **  *                *                 *             **
Ia    CCAAGQQWAIDNDE  CQEIPENGAQSDI  CRIAQRQ   CCISYLKEKS
        TD N M NQHRD   SLPYTSESKE--    MV E      HNQLE LH

Ib    CVAGVMGAKEGET  CGAEDNDT--        CGVSLYKQ CCD
        AT INL S P G   ASLHSYNSS        LETIFI R  H

Ic    CCGLGLRVRAEGQS CESNPNLGYP        CNHVMLS  CC
        M  KASL RD T   PIVMIS-Q          GL FRA
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Domain II

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          ****  *           *        *  *  ***  *         *  *                 *
IIx   DQDE CLMLPGEL      CQHL  CINTVGSYR  CA        CFPGFELQGDGRT   CR

IIa   QPNT CKDNGP        CRQV  CRVVGDTAM  CS        CFPGYAIMADGVS   CE
      LNDR  RGG           K Q    DT EVI              V  QLQS

IIy   DQDE CLMGTHD       CSWKQF CVNTLGSFY CVNHTVL   CAEGYILNAHRK    CV

IIb   DINE CVTDLHT       CTRAEH CVNTPGSFQ CYKALT    CEPGYVLTDGE-    CT
           I GSHN        RLGES  I  V   R  QRDSS      GT  EL EDNN     K

IIc   DVDE CVTGTHN       CQAGFS CQNTKGSFY CQARQR    CMDGFLQDPEGN    CV
           I    E  I      PPDFI      L   R RPKLQ     KS  I  AL       I

IId   DINE CTSLLEP       CRSGFS CINTVGSYT CQRNPLV   CGRGYHANEEGSE   CV
           L ISA         PV QT      E     K VPN      L      TR

IIe   DVNE CETGVHR       CGEGQL CYNLPGSYR CD        CKPGFQRDAFGRT   CI
           D   APPAEP    K HH   L S   F  E          A   YF GIS       V

IIf   DVNE CWVSPGRL      CQHT   CENTPGSYR CS        CAAGFLLAADGKH   CE
           I    QRY      G K          FH            S   R SV  RS

IIg   DVNE CETRR         CSQE   CANIYGSYQ CY        CRQGYQLAE-DGHT  CT
           LNSP                 V                   R    SDV  V  E

IIh   DIDE CAQGAGIL-     CTFR   CVNVPGSYQ CA        CPEQGYTMMANGRS  CK
           LPT GHI       SY     I I   F  S          SS  RLAP   N   Q

IIi   DLDE CALGTHN       CSEAET CHNIQGSFR CLRFD     CPPNYVRVSQTK    CE
           I    VT       IN     F          S E      E  R SAD  R      A
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Domain III

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RTTCQDITECQTSPARITHYQLNFQTGLLVPAHIFRIGPAPAFAGDTISLTITKGNEEGYFV
 LP HENQ   PRLPLS  Y H S P NIQ   VV  M  SS VP  SMQ A  A       F T

TRRLNAYTGVVSLQRSVLEPRDFALDVEMKLWRQGSVTTFLAKMYIFFTTFAP-COOH
 KVSHHS    A TKPIP       LL T K D Y H T SS V  LF  VSAEL-COOH
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Figure 3. Sequence alignment of similar repeats in domains I and II of fibulin-2 and comparison with the sequence of mouse fibulin-1. The assignment of repeats is correlated to those in Figs. 1 and 2 and include in addition the single copy of domain III. The sequences shown on top are from fibulin-2 with substitutions found in fibulin-1 indicated underneath. Dashes indicate gaps introduced for optimal alignment of the two fibulin sequences. Asterisks on top of domain I and II sequences indicate residues conserved in most of the repeats, including several conservative substitutions. They include all residues considered to be essential for calcium ligation in domain II, except for the repeat IIa.

Sunnerhagen et al., 1992), and all belong to the type II consensus motif (Handford et al., 1991).

Sequence comparison of those domains shared by fibulin-1 and -2 (Fig. 3) showed a low identity of 26% for domain I and required several gaps to be introduced for cysteine alignment. Identity was increased to 55% in domain II with all the cysteines and residues required as calcium ligands being conserved. Domain III showed 43% identity when compared to fibulin-1 variant C but no similarity to the corresponding variants B and D (see Fig. 2). In addition, comparison of the link regions failed to show any similarity. Taken together, the average identity is 43% for those regions common to both fibulin isoforms (666 residues).

Recombinant Expression of Fibulin-2

Several stable 293 cell clones were obtained after transfection with full-length fibulin-2 cDNA in an expression vector containing transcriptional control regions of the human cytomegalovirus. These 293-cell clones showed a single band in Northern blots similar in size to that of fibroblasts, and which was not detectable in the parent 293 cells. Examination of serum-free cell culture medium by SDS electrophoresis under reducing conditions revealed a major ~200-kD band unique to those clones and, occasionally, some smaller degradation products that blotted with an antiserum against fibulin-2 (data not shown). These findings were confirmed by radioimmunoassay of the media obtained from various clones (see below), which demonstrated 1–18 μg/ml of fibulin-2. Less than 0.02 μg/ml was detected in 293 cells.

A single clone (MK9-41) which showed high production and minimal degradation was used for large scale purification of fibulin-2. After molecular sieve chromatography of ammonium sulfate–precipitated material, the protein eluted in a distinct peak shortly after the void volume of a Sepharose CL-6B column. Some fibulin-2 was also detected in a peak emerging with the void volume and probably represents small amounts of aggregated protein. Fibulin-2 of >95% electrophoretic homogeneity was then obtained by Mono Q anion exchange chromatography, where the protein eluted in a broad range of the NaCl gradient (0.4–0.5 M). Reduced fibulin-2 consists of a single electrophoretic band with an apparent molecular mass of 195 kD (Fig. 4, lane 3). A major single band >400 kD was observed prior to reduction (Fig. 4, lane 1) indicating that fibulin-2 is secreted in the
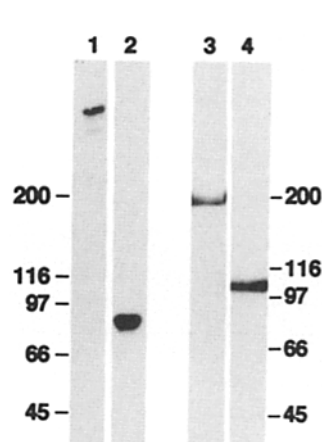
to fibulin-1 (Fig. 2), including domain I, which consists of three 35-residue motifs with a regular pattern of six cysteines similar to that of anaphylatoxins (Belt et al., 1984; Wetsel et al., 1984, 1987; de Bruijn and Fey, 1985). Because subdomain Ib lacks one cysteine and two are missing in Ib of fibulin-1 (Fig. 3), the same disulfide bridge as suggested from the structure of anaphylatoxin C3a (Huber et al., 1980) would be eliminated in both cases. Domain I is connected by a 50-residue link to the first EGF-like repeat IIx of domain II and is followed after another link (34 residues) by 10 more EGF-like repeats (IIa–i, y) and the COOH-terminal domain III. All these structures, except the repeats IIx and IIy and the first link, have homologous counterparts in fibulin-1. 10 of the EGF-like repeats of fibulin-2 (Fig. 3) possess the consensus sequence DX(D/N)ECXnCXnCXNX(F/Y)XC, indicating a calcium binding site (Rees et al., 1988; Selander-

Figure 4. Electrophoresis pattern of recombinant fibulin-2 in comparison to recombinant fibulin-1. Samples used were recombinant fibulin-2 (lanes *1* and *3*) and recombinant fibulin-1 (lanes *2* and *4*). They were analyzed under nonreducing conditions (lanes *1* and *2*) or after reduction (lanes *3* and *4*). Runs were calibrated with nonreduced (left margin) or reduced (right margin) globular proteins with migration position indicated by their molecular masses (kD).

form of a defined oligomer. Recombinant fibulin-1, however, consists of a single 85-kD band which shifts to a 105-kD migration position after reduction (Fig. 4, lanes 2 and 4) as previously observed for tissue-derived forms of the protein (Argraves et al., 1989; Kluge et al., 1990).

In addition to the major 200-kD band, several other clones showed a distinct 160-kD band, which in some purified batches, even predominated. Several more distinct although minor bands (100–140 kD) could be detected occasionally and may have arisen during purification. These smaller variants were also connected into oligomers by disulfide bonds. Because these smaller fibulin-2 variants cannot be the products of mRNA splicing, they must have been generated by endogenous proteolysis.

Structural and Immunological Properties

Edman degradation of the fibulin-2 195-kD band demonstrated the NH_2-terminal sequence VPWQDXTGA, in agreement with the signal peptidase cleavage site predicted from the cDNA sequence (Fig. 1). Finding the same NH_2-terminal sequence as was found for the 160-kD band would indicate that proteolytic shortening starts from the COOH-terminal end. Rotary shadowing of disulfide-bonded fibulin-2 revealed particles of low contrast showing a characteristic three arm structure composed of thin rods (Fig. 5). The average length of the rods was 40–45 nm, but smaller rods were also noted which could represent artifacts of degradation or could reflect imperfect metal decoration. The connecting structure frequently appears as a globular domain. Smaller
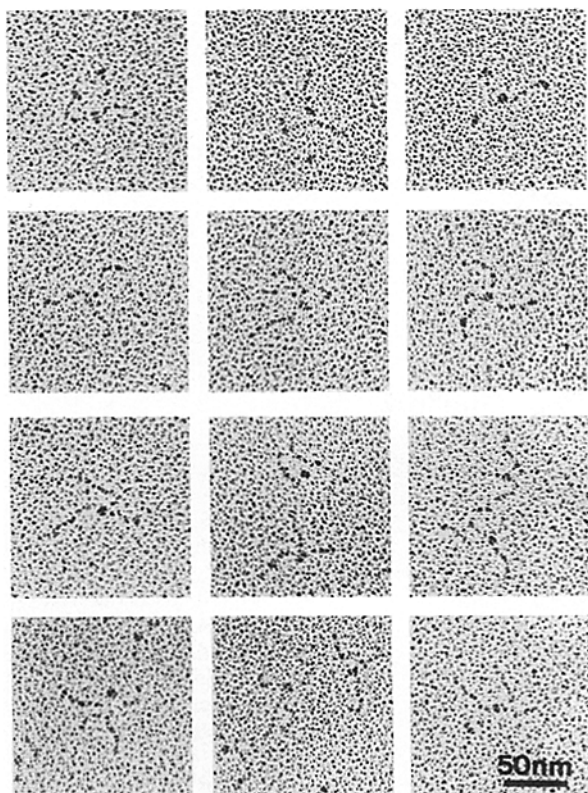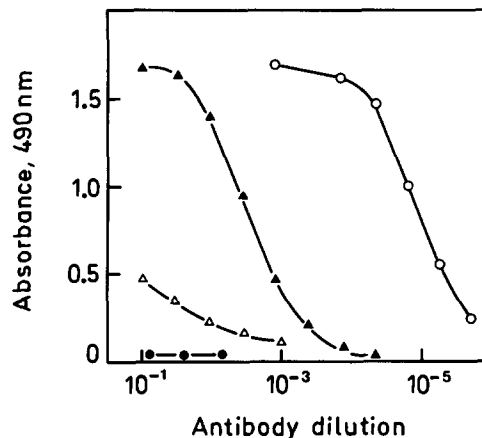

Figure 6. ELISA binding profiles of antisera against fibulin-1 and -2. The antibodies used were an antiserum against recombinant fibulin-2 (o, Δ) and affinity-purified antibodies against tissue-derived fibulin-1 (●, ▲). They were titrated against recombinant fibulin-2 (o, ●) and recombinant fibulin-1 (Δ, ▲).

globular structures were seen occasionally at the free ends of the strands. We interpret this major structure as an oligomer of three fibulin-2 chains in which only a limited portion of the chains are required for association. Other particles of fibulin-2 appeared as dimers or tetramers, or occurred in large aggregates with an undefined structure.

A rabbit antiserum was raised against fibulin-2 which showed in ELISA a high titer for fibulin-2 and a 1,000-fold lower binding to fibulin-1 (Fig. 6). A similar low binding was observed for recombinant nidogen but no reaction occurred

Figure 5. Visualization of individual particles of fibulin-2 by rotary shadowing. Note a frequent characteristic three-arm structure.
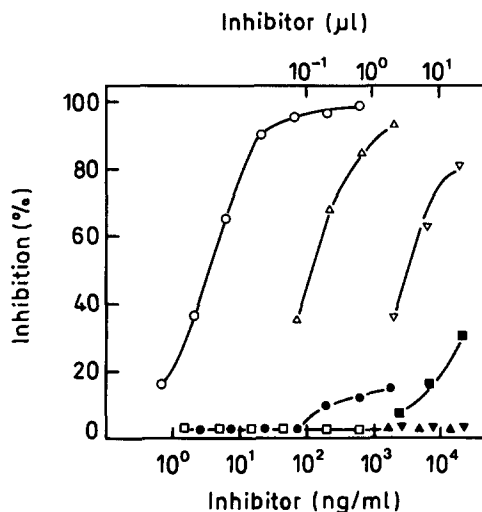

Figure 7. Analysis of fibulin-2 and biological samples by radioimmuno-inhibition assay. The test consisted of fixed concentrations of antiserum to fibulin-2 and ^{125}I-labeled fibulin-2. Purified protein inhibitors used at various concentrations (bottom scale, ng/ml) were recombinant fibulin-2 (o), fibulin-1 (□), and reduced and alkylated fibulin-2 (●). Biological fluids used as inhibitors were culture medium from fibulin-2 clone MK9-21 (Δ), parental 293 cells (▲), fibroblasts (▽), F9 cells (▼), and normal mouse serum (■). The amounts (in μl) added to 0.2 ml inhibitor solution are shown on the top scale.

Pan et al. Structure and Expression of Fibulin-2

1273

with laminin–nidogen complex, perlecan, BM-40, fibronectin, or vitronectin. In contrast, antisera or affinity-purified antibodies against tissue-derived fibulin-1 (Fig. 6) showed a distinct reaction with recombinant fibulin-1 but no reaction with fibulin-2. This suggests that the antisera should be able to differentiate between the two isoforms of fibulin in complex biological specimens.

In addition, the antiserum against fibulin-2 showed high titer in radioimmunoassay, allowing us to establish a sensitive inhibition test (IC_{50} = 4 ng/ml) for the quantitative determination of the antigen (Fig. 7). Inhibition profiles with identical slopes produced by fibulin-2 and culture medium from fibulin-2 clones and fibroblasts indicate that the antigens present in the biological samples share the same epitopes (Timpl, 1982). Mouse serum, however, was only a weak inhibitor, indicating a fibulin-2 concentration of less than 20 ng/ml. The inhibition assay was specific because fibulin-1, even at 1,000-fold excess, showed no activity. The inhibitory activity of reduced and alkylated fibulin-2 was decreased to low levels indicating that most of the epitopes involved depend on an intact conformation.

Expression and Localization of the Fibulin Isoforms in Tissues

Initial studies were performed with cultured cells using Northern blots and radioimmunoassay determinations of proteins secreted in the culture medium (Fig. 7). Fibulin-2 was detected in four different mouse fibroblast media at an average concentration of 260 ± 130 ng/ml but was not detected in the medium of mouse teratocarcinoma cell line F9, parietal endoderm cells PYS-2, and B16F10 melanoma cells (<20 ng/ml). Northern blot analysis of total RNA from mouse fibroblasts showed hybridization to a broad band at

the position of 28S ribosomal RNA (Fig. 8 A). This is consistent with the existence of at least two different mRNA transcripts due to the presence or absence of a single EGF-like repeat (IIy). Fibulin-2 mRNA was not expressed in F9 cells (data not shown) in contrast to the previous observation for fibulin-1 (Pan et al., 1993).

The expression of fibulin-2 was also examined by Northern blotting using poly A-rich RNA from various adult mouse tissues (Fig. 8 B). The fibulin-2 probe detected a 4.5-kb mRNA, in good agreement with the size of the full-length cDNA. However, the cDNA also detected another transcript of ~7.0 kb in some tissues. It is not clear whether or not the 7.0-kb band is transcribed from the same gene. The strongest hybridization was observed in heart tissue and much weaker signals were found in lung, liver, skeletal muscle, kidney, brain, spleen, and testis tissue. It was noted the 7-kb transcript was stronger than the 4.5-kb band in kidney and brain tissue. The tissue expression pattern was compared to that of fibulin-1 (Fig. 8 B) which is characterized by 2.3- and 2.7-kb transcripts (Argraves et al., 1990; Pan et al., 1993). Both fibulin-1 and -2 mRNAs were present at very high levels in heart tissue. However, more fibulin-1 mRNA than fibulin-2 mRNA was found in lung, testis, kidney, and brain tissue.

A comparative study of the tissue localization of the two isoforms of fibulin was performed by indirect immunofluorescence. Identical staining patterns were observed for skeletal muscle and heart tissue outlining the extracellular space that surrounds the muscle fibers (Fig. 9 a and b). In the cornea, both fibulin-1 and fibulin-2 are found along Bowman's and Descemet's membrane (Fig. 9 c). Staining for fibulin-1 was strong in the stromal compartment. In contrast, strong deposits of fibulin-2 could be detected in the squamous epithelium on top of Bowman's membrane, while only little staining was found in the stroma. Kidney tissue con-
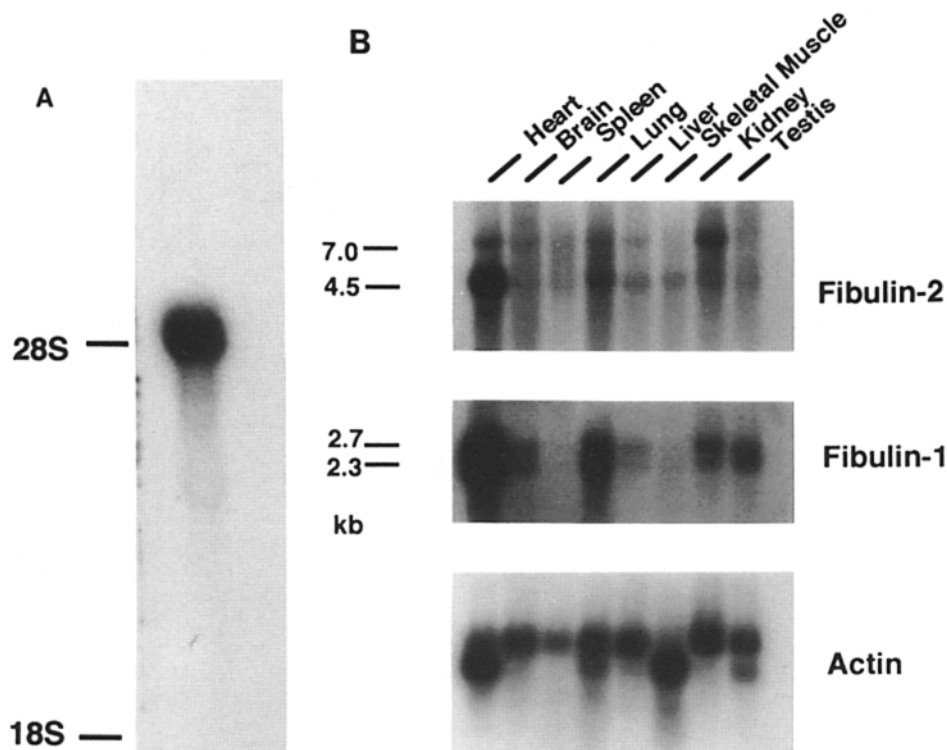

Figure 8. Northern blots of RNA from mouse fibroblasts and tissues. (*A*) 10 μg of total RNA from mouse fibroblasts was probed with fibulin-2 cDNA clone MK9. (*B*) Northern blot analysis of poly A-enriched RNAs from mouse tissues. Each lane contained 2 μg of poly A-enriched RNA and was probed sequentially with fibulin-2 cDNA (MK9), fibulin-1 cDNA (MK24) (Pan et al., 1993), and human actin cDNA.
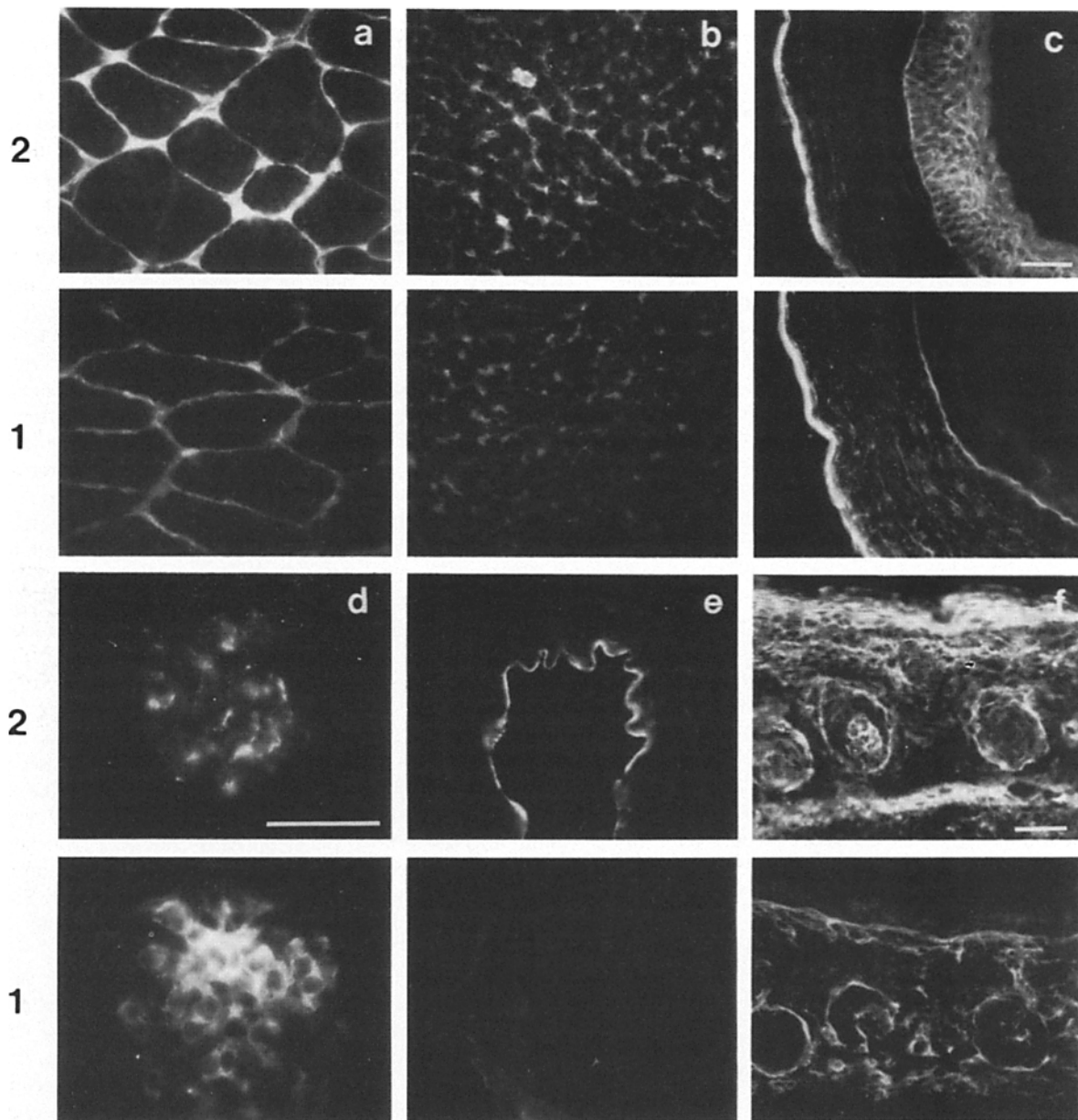
Figure 9. Immunofluorescence staining pattern of various mouse tissues by antisera specific for fibulin isoforms. Staining in row 1 was with affinity-purified anti-fibulin-1 and in row 2 with anti-fibulin-2. Two consecutive tissue sections are illustrated under *a–f*: (*a*) Skeletal muscle, (*b*) heart muscle, (*c*) cornea with Descement's membrane on the left, (*d*) kidney showing a single glomerulus, (*e*) aorta in cross section, (*f*) skin with the epidermis on top and the subcutis starting at the bottom region. Similar tissue sections were also incubated with normal rabbit serum which yielded negative results except for a very weak staining of the epidermis (data not shown). Bars, 40 μm.

tained both isoforms of fibulin as illustrated for a single glomerulus in Fig. 9 *d*. Both antisera showed strong staining of the mesangium. The antiserum against fibulin-1 showed a distinct reaction with the glomerular loops (Fig. 9 *d*), and a weak staining of tubular basement membranes (not shown). The latter observation was confirmed previously by immunogold staining (Kluge, 1990). Examination of kidney sections at low magnification demonstrated a strong reaction for fibulin-2 but not for fibulin-1 in the wall of small vessels. A similar difference was observed for aorta with strong staining for fibulin-2 in the subintimal layer and very faint reactions in the media and adventitia (Fig. 9 *e*). No signifi-

cant staining of aorta could be observed for fibulin-1. Similarly, fibulin-2, but not fibulin-1, could be detected around portal veins in the liver, while no staining was observed in the perisinusoidal space (not shown). The strongest reactions for fibulin-2 were observed in the skin including the epidermal cell layer, the dermis, and the adipose tissue in the subcutis (Fig. 9 *f*). A distinct staining was also seen around the glassy membranes and the keratinocytes of hair follicles. Staining for fibulin-1 was also distinct but mainly restricted to the glassy membranes, the epidermal–dermal junction, and some dermal structures underneath this junction which could represent small capillaries.

Discussion

Sequence analysis, recombinant expression, and immunological assays demonstrate that fibulin-2 is a new extracellular matrix protein. The closest relative seems to be fibulin-1, with 43% sequence identity between the domains shared by the two proteins. This level of identity is in the range observed for other protein isoforms like the diverse laminin chains, which also contain multiple EGF-like repeats (Engel, 1993). We suggest classification of the fibulins into a novel protein family and suggest use of arabic numbers for their distinction as used with the thrombospondins (Bornstein, 1992). Many consecutive EGF-like repeats with calcium-binding motifs are the hallmark of fibrillin (Lee et al., 1991; Maslen et al., 1991) and TGF-$\beta 1$ binding protein (Kanzaki et al., 1990) but are less abundant in nidogen (Mann et al., 1989) and EGF precursor (Scott et al., 1983). The multiple repeats in these proteins, however, are joined to domains different from those found in the fibulins. In addition, several *Drosophila* and *Caenorhabditis elegans* proteins (notch, lin-12, and others) contain tandem arranged EGF-like repeats and play a role in development (Kelley et al., 1987; Yochem and Greenwald, 1989; Bork, 1991). The common structural motifs suggest that these proteins may have originated from common ancestral precursors by exon shuffling and together represent a superfamily.

EM of recombinant fibulin-2 demonstrates a trimeric rod structure connected by disulfide bonds. A similar structure but with large, calcium-dependent globular domains at the distal ends was described for thrombospondin-1 (Lawler et al., 1985). The two proteins show only a low similarity in sequence and domain structure (Lawler and Hynes, 1986). It is not presently known whether or not other isoforms of thrombospondin (Bornstein, 1992) have similar structures. In contrast, fibulin-1 has the shape of a single rod of ~ 30 nm length (Kluge, 1990). The rods of fibulin-2 are longer (40–45 nm) and very likely include the 11 EGF-like repeats of domain II. The predicted length for a row of such repeats is about 25 nm (Engel, 1989), suggesting that other domains (Nb, I and III) are included in the rod structure. The reduced chain of fibulin-2 and a truncated 160-kD form share the same NH_2-terminal sequence, thus indicating that domain Na contributes the disulfide bonds for the connection of the rods and is part of the central globular domain. A disulfide-linked NH_2-terminal connection has also been identified for thrombospondin-1.

The molecular mass of the fibulin-2 chain, based on the sequence and assuming substitutions with typical N-linked oligosaccharides, was calculated to be ~ 142 kD. The mass determined from electrophoretic migration, however, was 195 kD. A similar difference between calculated (82 kD) and observed (105 kD) molecular mass was found for fibulin-1, indicating either substitution with unusually large oligosaccharides, or an anomalous electrophoretic mobility common to both fibulins, which should be further studied by ultracentrifugation. Calcium binding has been shown only for fibulin-1 (Argraves et al., 1990; Kluge et al., 1990), but the number of occupied sites and their affinities are not yet known. The binding of fibulin-1 to laminin or nidogen is dependent on calcium and completely abolished by EDTA (Pan et al., 1993). Preliminary studies with fibulin-2 show that its binding to fibronectin and some other ligands is also calcium dependent (Sasaki, T., and R. Timpl, unpublished results), suggesting that some of the putative calcium-binding motifs in domain II of fibulin-2 are active in providing calcium coordination sites.

A low immunological cross-reactivity was observed between the two fibulins which is presumably accounted for by the relatively low sequence identity and further differences in the domain structure. This allows us to differentiate the two proteins by radioimmuno- and other immunological assays in various biological samples. The serum concentration of fibulin-2 is low and comparable with those of most other extracellular matrix proteins (Risteli and Risteli, 1987). A serum concentration at at least 1,000-fold higher (20–30 $\mu g/ml$) has been found for fibulin-1 (Argraves et al., 1990; Kluge et al., 1990) suggesting different cellular origins of the circulating fibulin isoforms. We have shown by Northern blots that liver cells are an unlikely source of serum fibulin-1, although hepatocytes are the main producers of serum fibronectin (Hynes, 1990).

Fibroblasts are the only cells examined so far that produce both isoforms of fibulins as shown in this and previous studies (Argraves et al., 1990; Kluge et al., 1990). Fibulin-2 was not detected by radioimmunoassay in several mouse tumor cell lines, but a substantial secretion of fibulin-1 (100–460 ng/ml) was observed (Kluge et al., 1990). An overlapping, but not identical, tissue expression pattern of both fibulins was also observed by Northern analyses of RNAs from cultured cells and tissues. It appears that the expression of fibulin-2 is more restricted, and the regulation of the expression of the two fibulin isoforms is not strictly coordinated. Of particular interest is the high expression of both fibulin mRNAs in heart tissue. Fibulin-1 has been shown to be enriched in the endocardial cushion tissue during heart development (Spence et al., 1992; Zhang et al., 1993). The expression of fibulin-2 in developing heart tissue remains to be investigated.

Differences are noted in the tissue localization of the two fibulins by indirect immunofluorescence. Fibulin-2, but not fibulin-1, is detected in the keratinocyte cell layer of the epidermis and hairs, the epithelial cell layer of cornea, the aortic intima, and around small vessels in kidney and liver. However, staining of fibulin-1 seems to be much stronger than fibulin-2 in the corneal stroma and glomerular basement membranes. Both isoforms colocalize in several other anatomical compartments such as the perimysium of skeletal muscle, the matrix surrounding myocytes, the renal mesangium, and the two basement membranes of the cornea, but the latter interpretation still needs confirmation by immuno electronmicroscopy. Together the data demonstrate that fibulin-2 is a typical extracellular matrix protein, which can be a component of both basement membranes and other connective tissues.

References

Argraves, W. S., K. Dickerson, W. H. Burgess, and E. Ruoslahti. 1989. Fibulin, a novel protein that interacts with the fibronectin receptor β subunit cytoplasmic domain. *Cell.* 58:623–629.

Argraves, W. S., H. Tran, W. H. Burgess, and K. Dickerson. 1990. Fibulin is an extracellular matrix and plasma glycoprotein with repeated domain structure. *J. Cell Biol.* 111:3155–3164.

Balbona, K., H. Tran, S. Godyna, K. C. Ingham, D. K. Strickland, and W. S. Argraves. 1992. Fibulin binds to itself and to the carboxyl-terminal heparin-binding region of fibronectin. *J. Biol. Chem.* 267:20120–20125.

Baron, M., D. G. Norman, and D. Campbell. 1991. Protein modules. *Trends Biochem. Sci.* 16:13–17.

Beck, K., I. Hunter, and J. Engel. 1990. Structure and function of laminin: anatomy of a multidomain glycoprotein. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:148–160.

Belt, K. T., M. C. Carroll, and R. R. Porter. 1984. The structural basis of the multiple forms of human complement component C4. *Cell.* 36:907–914.

Bork, P. 1991. Shuffled domains in extracellular proteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 286:47–54.

Bornstein, P. 1992. Thrombospondins: structure and regulation of expression. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:3290–3299.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

de Bruijn, M. H. L., and G. H. Fey. 1985. Human complement component C3: cDNA coding sequence and derived primary structure. *Proc. Natl. Acad. Sci. USA.* 82:708–712.

Dietz, H. C., G. R. Cutting, R. E. Pyeritz, C. L. Maslen, L. Y. Sakai, G. M. Corson, E. G. Puffenberger, A. Hamosh, E. J. Nanthakumar, S. M. Curristin, G. Stetten, D. A. Myers, and C. A. Francomano. 1991. Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature (Lond.).* 352:337–339.

Dietz, H. C., R. E. Pyeritz, E. G. Puffenberger, R. J. Kendzior, G. M. Corson, C. L. Maslen, L. Y. Sakai, C. A. Francomano, and G. R. Cutting. 1992. Marfan phenotype variability in a family segregating a missense mutation in the epidermal growth factor-like motif of the fibrillin gene. *J. Clin. Invest.* 89:1674–1680.

Engel, J. 1989. EGF-like domains in extracellular matrix proteins: Localized signals for growth and differentiation? *FEBS (Fed. Am. Soc. Exp. Biol.) Lett.* 251:1–7.

Engel, J. 1993. Structure and function of laminin. *In* Molecular and Cellular Aspects of Basement Membranes, D. H. Rohrbach, and R. Timpl, editors. Academic Press, San Diego, CA. 147–176.

Fox, J. W., U. Mayer, R. Nischt, M. Aumailley, D. Reinhardt, H. Wiedemann, K. Mann, R. Timpl, T. Krieg, J. Engel, and M.-L. Chu. 1991. Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3137–3146.

Handford, P. A., M. Baron, M. Mayhew, A. Willis, T. Beesley, G. G. Brownlee, and I. D. Campbell. 1990. The first EGF-like domain from human factor IX contains a high-affinity calcium binding site. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:475–480.

Handford, P. A., M. Mayhew, M. Baron, P.R. Winship, I. D. Campbell, and G. G. Brownlee. 1991. Key residues involved in calcium-binding motifs in EGF-like domains. *Nature (Lond.).* 351:164–167.

Huber, R., H. Scholze, E. P. Paques, and J. Deisenhofer. 1980. Crystal structure analysis and molecular model of human C3a anaphylatoxin. *Hoppe-Seyler's Z. Physiol. Chem.* 361:1389–1399.

Hynes, R. O. 1990. Fibronectins. Springer-Verlag, New York.

Johnson, G. D., R. S. Davidson, K. C. McNamee, G. Russel, D. Goddwin, and E. J. Holborow. 1982. Fading of immunofluorescence during microscopy: a study of its phenomena and its remedy. *J. Immunol. Methods.* 55:231–242.

Kanzaki, T., A. Olofsson, A. Moren, C. Wernstedt, U. Hellman, K. Miyazono, L. Claesson-Welsh, and C.-H. Heldin. 1990. TGF-β1 binding protein: a component of the large latent complex of TGF-β1 with multiple repeat sequences. *Cell.* 61:1051–1061.

Kelley, M. R., S. Kidd, W. A. Deutsch, and M. W. Young. 1987. Mutations altering the structure of epidermal growth factor-like coding sequences at the *Drosophila* notch locus. *Cell.* 5:539–548.

Kluge, M. 1990. Isolierung und Charakterisierung eines neuen Basalmembranproteins aus dem EHS-Maustumor. Doctoral thesis. University Munich, Munich.

Kluge, M., K. Mann, M. Dziadek, and R. Timpl. 1990. Characterization of a novel calcium-binding 90-kDa glycoprotein (BM-90) shared by basement membranes and serum. *Eur. J. Biochem.* 193:651–659.

Lawler, J., and R. O. Hynes. 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* 103:1635–1648.

Lawler, J., L. H. Derrick, J. E. Conolly, J. H. Chen, and F. C. Chao. 1985. The structure of human platelet thrombospondin. *J. Biol. Chem.* 260:3762–3772.

Lee, B., M. Godfrey, E. Vitale, H. Hori, M.-G. Mattei, M. Sarfarazi, P.

Tsipouras, F. Ramirez, and D. W. Hollister. 1991. Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. *Nature (Lond.).* 352:330–334.

Mann, K., R. Deutzmann, M. Aumailley, R. Timpl, L. Raimondi, Y. Yamada, T. Pan, D. Conway, and M.-L. Chu. 1989. Amino acid sequence of mouse nidogen, a multidomain basement membrane protein with binding activity for laminin, collagen IV and cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:65–72.

Maslen, C. L., G. M. Corson, B. K. Maddox, R. W. Glanville, and L. Y. Sakai. 1991. Partial sequence of a candidate gene for the Marfan syndrome. *Nature (Lond.).* 352:334–337.

Mayer, U., R. Nischt, E. Pöschl, K. Mann, K. Fukuda, M. Gerl, Y. Yamada, and R. Timpl. 1993. A single EGF-like motif of laminin is responsible for high affinity nidogen binding. *EMBO (Eur. Mol. Biol. Organ.) J.* 12: 1879–1885.

Nischt, R., J. Pottgiesser, T. Krieg, U. Mayer, M. Aumailley, and R. Timpl. 1991. Recombinant expression and properties of the human calcium-binding extracellular matrix protein BM-40. *Eur. J. Biochem.* 200:529–536.

Oldberg, A., A. Franzen, and D. Heinegard. 1988. The primary structure of a cell-binding bone sialoprotein. *J. Biol. Chem.* 263:19430–19432.

Pan, T.-C., M. Kluge, R.-Z. Zhang, U. Mayer, R. Timpl, and M.-L. Chu. 1993. Sequence of extracellular mouse protein BM-90/fibulin and its calcium-dependent binding to other basement membrane ligands. *Eur. J. Biochem.* 215:733–740.

Panayotou, G., P. End, M. Aumailley, R. Timpl, and J. Engel. 1989. Domains of laminin with growth-factor activity. *Cell.* 56:93–101.

Paulsson, M., M. Aumailley, R. Deutzmann, R. Timpl, K. Beck, and J. Engel. 1987a. Laminin-nidogen complex: extraction with chelating agents and structural characterization. *Eur. J. Biochem.* 166:11–19.

Paulsson, M., P. D. Yurchenco, G. C. Ruben, J. Engel, and R. Timpl. 1987b. Structure of low density heparan sulfate proteoglycan isolated from a mouse tumor basement membrane. *J. Mol. Biol.* 197:297–313.

Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA.* 85:2444–2448.

Persson, E., M. Selander, S. Linse, T. Drakenberg, A.-K. Öhlin, and J. Stenflo. 1989. Calcium binding to the isolated β-hydroxyaspartic acid-containing epidermal growth factor-like domain of bovine factor X. *J. Biol. Chem.* 264:16897–16904.

Rees, D. J. G., I. M. Jones, P. A. Handford, S. J. Walter, M. P. Esnouf, K. J. Smith, and G. G. Brownlee. 1988. The role of β-hydroxyaspartate and adjacent carboxylate residues in the first EGF domain of human factor IX. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2053–2061.

Risteli, L., and J. Risteli. 1987. Analysis of extracellular matrix proteins in biological fluids. *Methods Enzymol.* 145:391–411.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Scott, J., M. Urdea, M. Quiroga, R. Sanchez-Pescador, N. Fong, M. Selby, W. J. Rutter, and G. I. Bell. 1983. Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins. *Science (Wash. DC).* 221:236–240.

Selander-Sunnerhagen, M., M. Ullner, E. Persson, O. Teleman, J. Stenflo, and T. Drakenberg. 1992. How an epidermal growth factor (EGF)-like domain binds calcium. High resolution NMR structure of the calciumform of the NH2-terminal EGF-like domain in coagulation factor X. *J. Biol. Chem.* 267:19642–19649.

Spence, S. G., W. S. Argraves, L. Walters, J. E. Hungerford, and C. D. Little. 1992. Fibulin is localized at sites of epithelial-mesenchymal transitions in the early avian embryo. *Dev. Biol.* 151:473–484.

Spring, J., K. Beck, and R. Chiquet-Ehrismann. 1989. Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell.* 59:325–334.

Timpl, R. 1982. Antibodies to collagens and procollagens. *Methods Enzymol.* 82:472–498.

von Heijne, G. 1985. Signal sequences. The limits of variation. *J. Mol. Biol.* 184:99–105.

Wetsel, R. A., A. Lundwall, F. Davidson, T. Gibson, B. F. Tack, and G. H. Fey. 1984. Structure of murine complement component C3: II. Nucleotide sequence of cloned complementary DNA coding for the α chain. *J. Biol. Chem.* 259:13857–13862.

Wetsel, R. A., R. T. Ogata, and B. F. Tack. 1987. Primary structure of the fifth component of murine complement. *Biochemistry.* 26:737–743.

Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13:281–292.

Yochem, J., and I. Greenwald. 1989. Glp-1 and lin-12, genes implicated in distinct cell-cell interactions in *C. elegans*, encode similar transmembrane proteins. *Cell.* 58:553–563.

Zhang, H.-Y., M. Kluge, R. Timpl, M.-L. Chu, and P. Ekblom. 1993. The extracellular matrix glycoproteins BM-90 and tenascin are expressed in the mesenchyme at sites of endothelial-mesenchymal conversion in the embryonic mouse heart. *Differentiation.* 52:211–220.